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Substrate and Inhibitor Profile of BACE (β-Secretase) and Comparison with Other Mammalian Aspartic Proteases*

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The full-length and ectodomain forms of β-site APP cleavage enzyme (BACE) have been cloned, expressed in Sf9 cells, and purified to homogeneity. This aspartic protease cleaves the amyloid precursor protein at the β-secretase site, a critical step in the Alzheimer's disease pathogenesis. Comparison of BACE to other aspartic proteases such as cathepsin D and E, napsin A, pepsin, and renin revealed little similarity with respect to the substrate preference and inhibitor profile. On the other hand, these parameters are all very similar for the homologous enzyme BACE2. Based on a collection of decameric substrates, it was found that BACE has a loose substrate specificity and that the substrate recognition site in BACE extends over several amino acids. In common with the aspartic proteases mentioned above, BACE prefers a leucine residue at position P1. Unlike cathepsin D etc., BACE accepts polar or acidic residues at positions P2′ and P1 but prefers bulky hydrophobic residues at position P3. BACE displays poor kinetic constants toward its known substrates (wild-type substrate, SEVKM↓DAEFR, $K_m = 7$ μM, $K_{cat} = 0.002$ s^{-1}; Swedish mutant, SEVNL↓DAEFR, $K_m = 9$ μM, $K_{cat} = 0.02$ s^{-1}). A new substrate (VVEVDA↓AVTP, $K_m = 1$ μM, $K_{cat} = 0.004$) was identified by serendipity.

Alzheimer's disease is characterized by the extracellular deposition of insoluble amyloid plaques. The main component of amyloid plaques is the 39–43-amino acid β-amyloid peptide (Aβ),[1] which derives from a larger protein precursor (amyloid precursor protein, APP). Aβ is excised from APP by the sequential action of two proteases known, respectively, as β-secretase, which cuts amino-terminal to Aβ, and γ-secretase, which cleaves at the carboxyl terminus. Several reports appeared recently describing the cloning and characterization of β-secretase (1–5). This protein, designated Asp-2, BACE, or memapsin 2, according to the laboratory in which it was discovered, is a novel transmembrane aspartic protease that cleaves APP at the β-secretase site. BACE possesses all the characteristics expected for β-secretase in terms of substrate preference, pH optimum for activity, tissue distribution, and subcellular localization. In addition, two recent reports indicate that Aβ levels in the brains of BACE knockout mice are reduced by more than 90% compared with control mice (6, 7). In addition to cleaving APP at the β-secretase site, BACE cuts APP further downstream within the amyloid region (between Tyr-10 and Glu-11 of Aβ), generating a truncated form of Aβ that is probably still amyloidogenic (3, 8). Parallel to the discovery of BACE, a second, homologous transmembrane aspartic protease termed Asp-1, BACE2, memapsin 1, or down region aspartic protease was reported (4, 5, 9, 10). Preliminary analysis of BACE2 indicated that it can also function as a β-secretase *in vitro* (8, 9).

In seeking to develop a disease-modifying therapy for Alzheimer's Disease, BACE presents itself as an ideal drug target. It belongs to a well understood class of protease where inhibitors have previously been developed for therapeutic use (renin, human immunodeficiency virus protease). Two different peptidomimetic inhibitors of BACE with nanomolar activity have already been described (3, 11). In addition, an x-ray crystal structure has been published (12), which should facilitate rational design of new inhibitors. However, because the treatment of Alzheimer's disease will be a long term therapy, a β-secretase inhibitor has to be very selective. Aspartic proteases are widely distributed in the body. BACE2, for instance, is found at low levels in most peripheral organs (13). Here we describe the enzymatic properties of BACE in comparison with its homologue, BACE2, and other relevant mammalian aspartic proteases, namely pepsin, cathepsin D, cathepsin E, napsin A and renin.

EXPERIMENTAL PROCEDURES

Cloning and Expression of BACE and BACE2

cDNAs encoding the aspartyl proteases BACE and BACE2 were modified by PCR in the 5′ non-coding region to optimize ribosomal recognition and at the 3′ end by adding sequences encoding 6×His residues to enable rapid purification of the recombinant proteins. Expression in Sf9 insect cells via recombinant baculovirus resulted in higher yields than expression in *Escherichia coli*, *Schizosaccharomyces pombe*, or HEK293 cells. Thus the cDNAs were cloned into the pFAST-BAC1 vector (Invitrogen) as *Bam*HI × *Xba*I fragments for expression in insect cells and the PCR products were confirmed by sequencing. After recombination into the baculovirus genome, the purified viral DNA was transformed into the insect cells. Sf9 cells were cultured at 27 °C in TC100 medium (BioWhittaker) with 5% (v/v) fetal calf serum. Virus stocks were generated with a titer of 1.5×10^9 plaque-forming units/ml. For large scale production of BACE and BACE2, 24-liter fermenters of Sf9 cells were infected with a multiplicity of infection of 1.

Preparation of Enzymes

BACE Ectodomain—The first step in purification of BACE ectodomain is immunoaffinity chromatography using a monoclonal antibody (BSC-1) generated in-house by Dr. M. Brockhaus. The antibody was generated by standard methods from a mouse immunized with BACE purified from *E. coli*. The specificity of BSC-1 (IgG1) was established by enzyme-linked immunosorbent assay and Western blot assays. Thus 5 liters of cell-free Sf9 fermentation broth was concentrated 10-fold by ultrafiltration and loaded directly onto a 1.6 × 4-cm BSC-1-Sepharose immunoaffinity column that had been equilibrated in 50 mM Tris-HCl,

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[1] The abbreviations used are: Aβ, β-amyloid peptide; APP, amyloid precursor protein; DMA, *N*,*N*-dimethylacetamide; Fmoc, fluorenylmethoxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*] pyridino-1-yl-methylmethanaminium hexafluorophosphate *N*-oxide; HPLC, high performance liquid chromatography; FRET, fluorescence resonance energy transfer; MES, 4-morpholinoethanesulfonic acid; BACE, β-site APP cleavage enzyme.

pH 7.2, 150 mM NaCl. The column was washed with several column volumes each of 50 mM Tris HCl, pH 7.2, 150 mM NaCl then 50 mM sodium citrate, pH 5.0, 500 mM NaCl. The column was eluted with 50 mM sodium citrate, pH 3.0. Fractions containing BACE were pooled, neutralized with sodium bicarbonate, then dialyzed against 50 mM Tris-HCl, pH 7.4. This material was passed over a Mono S HR 5/5 column (Amersham Biosciences, Inc.) that had been previously equilibrated in 50 mM Tris-HCl, pH 7.4. BACE, which is obtained in the column flow-through, was subsequently concentrated by ultrafiltration and chromatographed on a Superdex 200 HR 16/60 column (Amersham Biosciences, Inc.) in 50 mM Tris-HCl, pH 7.2, 100 mM NaCl. The purified BACE was stored at 4 °C.

Full-length BACE—50 g (wet weight) of Sf9 cells was suspended in 750 ml of phosphate-buffered saline, 2% Triton X-100 and homogenized with a hand-held glass homogenizer. The homogenate was stirred on ice for 30 min and then centrifuged at 100,000 × g for 20 min. The supernatant was adjusted to pH 8.0 and loaded on a 2.6 × 2.5-cm Ni²⁺-nitrilotriacetic acid-Sepharose column (Qiagen, Germany) that had been equilibrated in 50 mM sodium phosphate, 10 mM Tris, 100 mM NaCl, 0.1% Triton X-100, pH 8.0. The column was subsequently washed with this buffer and then with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.1% Triton X-100. The column was then eluted with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 200 mM imidazole, 0.1% Triton X-100 (10 column volumes). Pooled fractions containing full-length BACE were passed over a 5-ml HiTrap Q column (Amersham Biosciences, Inc.), and the unbound material was collected. This material was then diluted 10-fold into 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.1% Triton, and re-loaded onto a second 5-ml HiTrap Q column that had been equilibrated in 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.1% Triton X-100. The column was washed in this buffer and eluted with a gradient of 50 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.1% Triton X-100 (20 column volumes). Fractions containing BACE were pooled, dialyzed against 50 mM Tris-Cl, pH 8.0, 0.1% Triton X-100 and loaded on a Mono S HR5/5 column (Amersham Biosciences, Inc.). The unbound material, containing BACE, was pooled and stored at 4 °C.

Full-length BACE2—50 g (wet weight) of Sf9 cells were washed with 10 volumes of ice-cold phosphate-buffered saline and then suspended in 750 ml of phosphate-buffered saline, 1.5% dodecyl β-D-maltoside and homogenized with a hand-held glass homogenizer. The homogenate was stirred on ice for 30 min and then centrifuged at 100,000 × g for 20 min. The supernatant was adjusted to pH 8.0, and imidazole was added to a final concentration of 10 mM. The extract was then loaded on a 2.6 × 2.5-cm Ni²⁺-nitrilotriacetic acid-Sepharose column (Qiagen) that had been equilibrated in 50 mM sodium phosphate, 10 mM Tris 100 mM NaCl, 0.1% Triton X-100, pH 8.0. The column was subsequently washed with this buffer and then with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.1% Triton X-100. The column was then eluted with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 200 mM imidazole, 0.1% Triton X-100 (10 column volumes). BACE2-containing fractions were dialyzed against 25 mM sodium acetate, pH 4.5, 25 mM NaCl and further purified by affinity chromatography on P10-P4' StatVal-Sepharose, as described by Sinha *et al.* (3) for BACE. Protein concentration was determined using the BCA assay (Pierce).

Cathepsin D, Cathepsin E, Renin, Napsin, and Pepsin—Pepsin from hog stomach was purchased from Fluka (Buchs, Switzerland), and cathepsin D was purchased from Calbiochem. Recombinant mouse cathepsin E was a gift from Prof. John Kay and was purified according to Hill *et al.* (14). Recombinant human renin was purified according to Mathews *et al.* (15) and recombinant human napsin A was purified according to Schauer-Vukasinovic *et al.* (16).

Inhibitors

The BACE inhibitor P10-P4' StatVal (=Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe, where Sta is a statin transition state mimetic), originally described by Sinha *et al.* (3), was purchased from Bachem (Bubendorf, Switzerland). The BACE inhibitor OM99-2 (=Glu-Val-Asn-Mim-Ala-Glu-Phe, where Mim is a transition state mimetic containing a Leu-Ala motif and where the peptide bond is replaced by a CHOH-CH₂ group), originally described by Hong *et al.* (12) was synthesized in-house by Dr. Eric Kitas and co-workers. Saquinavir (Ro 31-8959) and Remikiren (Ro 42-5892) were taken from the Roche bulk products, and pepstatin was purchased from Fluka.

Fluorescence Resonance Energy Transfer (FRET) Assay

Synthesis and Analysis of Peptides—Peptides were synthesized on TentaGel S-Rinkamide resin (0.25 mmol/g, Rapp Polymer, Tübingen) following a standard procedure consisting of the repetition of the

following steps: 1) deprotection with 20% piperidine in *N,N*-dimethylacetamide (DMA); 2) washing with DMA, isopropyl alcohol, and DMA; 3) coupling with fluorenylmethoxycarbonyl (Fmoc)-protected amino acid using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridino-1-yl-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and diisopropylethylamine in *N*-methylpyrrolidone. Double coupling of the Fmoc-protected amino acid was performed by using *O*-(1,2-dihydro-2-oxo-1-pyridyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TPTU) and diisopropylethylamine in *N*-methylpyrrolidone. Boc-Lys-(dabsyl)-OH (where Boc is *tert*-butoxycarbonyl) was prepared by the reaction of Boc-Lys-OH with dabsyl chloride. Fmoc-Glu-Lucifer Yellow was obtained by condensation of the *N*-hydroxysuccinimide ester of Fmoc-Glu-(O*t*Bu)-OH (where *t*Bu is *tert*-butyl) with Lucifer Yellow and subsequent treatment with trifluoroacetic acid; 4) same washing as step 2. After the last cycle, the resins were treated with trifluoroacetic acid-/H₂O 95:5. Crude peptides were purified by reversed-phase HPLC using a Nucleosil-C₁₈ column (7 μm, 10 × 250 mm, Machery Nagel, gradient of H₂O and acetonitrile). Identity and purity of the peptides were analyzed by HPLC and electrospray ionization-mass spectroscopy (using a PE SCIEX, model API-100 liquid chromatograph).

Enzyme Assays—All enzyme assays were performed at 20 °C on a FLUOstar (BMG Lab Technologies, D-77656 Offenburg) using 96-well microtiter plates (DYNEX Microfluor 2, Chantilly, VA). The assay volume was 100 μl. Typically, inhibitors dissolved in dimethyl sulfoxide were added into a well followed by buffer and enzyme. The dimethyl sulfoxide concentration was kept below 4%. The enzymatic reaction was started by adding the substrate. pH and buffer conditions under which the experiments were carried out are indicated in the figure and table legends. The progression of the fluorescence increase was measured at $\lambda_{emission}$ = 520 nm with fluorescence excitation at $\lambda_{excitation}$ = 430 nm. Reaction kinetics were followed periodically for 30 min at various substrate concentrations. The detected signals were converted into moles of substrate hydrolyzed per second. Kinetic data were determined graphically from Lineweaver-Burk plots. It should be noted that data obtained by varying substrate concentrations had to be corrected for the effect of excess quenching capacity, which is typical of all the FRET substrates used here. Assays were performed at enzyme concentrations that warranted a linear progression of product formation.

Identification of the Cleavage Site of Soluble Substrates

The samples were dried in a Speed-Vac and then dissolved in 110 μl of 50% formic acid. 100 μl were subjected to reversed-phase HPLC using a AL12S05 column (5 μm, 5 × 100 mm, YMC, gradient of 1% formic acid and acetonitrile). Peak fractions were collected and dried again in a Speed-Vac. The peptide fragments were analyzed by electrospray ionization-mass spectroscopy (using a PE SCIEX, model API-100LC).

Identification of Cleavage Sites Using Combinatorial Libraries

Synthesis of Targeted Peptide Libraries—The FRET substrate combinatorial libraries were synthesized on PEGA₁₉₀₀ (copolymer of polyethylene glycol with a molecular weight of 1900 and acrylamide) resin via split synthesis after standard Fmoc chemistry using HATU as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA₁₉₀₀ resin using HATU in the presence of diisopropylethylamine in *N*-methylpyrrolidone. Double coupling was performed using *O*-(1,2-dihydro-2-oxo-1-pyridyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TPTU) in the presence of diisopropylethylamine in *N*-methylpyrrolidone. The synthesis was performed on a semi-automated shaking-vessel machine. For variable positions in the peptide sequences the resin was transferred in a Manual Multiple/Library Synthesizer (MULTIBLOCK®) and split into 19 portions. A different and single Fmoc-protected amino acid (except Fmoc-Cys-OH) was coupled to each of the 19 portions. Then the portions were mixed together and washed thoroughly. Depending on the library the splitting was either repeated or synthesis proceeded in a single portion. The last building block was *tert*-butoxycarbonyl-Lys-(dabsyl)-OH. The protecting groups were removed with trifluoroacetic acid:H₂O:TIS 95:3:2 for 2 h.

Screening of the Peptide Libraries—A part (~2000 beads) of the peptide library (either Lys(dabsyl)-SEV*XX*DAEFR-Glu(Gly-PEGA)-Lucifer Yellow or Lys(dabsyl)-SEV*XL*X*AEFR-Glu(Gly-PEGA)-Lucifer Yellow) was extensively washed with 100 mM sodium acetate, pH 5.0. Then 80 μl of the purified enzyme (ectodomain) was added to the resin in a final volume of 0.8 ml in the reaction buffer. After shaking for 18 h at room temperature, the resin was filtered off, and the beads were washed with the incubation buffer and 100 mM MES buffer, pH 4.8, containing 5% glycerol and 0.05% Triton X-100. The beads were in-

TABLE I
Soluble substrates and relative activity with BACE

The substrates are listed in the order of cleavage efficiency by BACE. An example of the structure with the decameric peptide sequence, the linker amino acids, the fluorophor, and the quencher is given in Fig. 2.

Substrate	Sequence	Substrate type	Cleavage by BACE
			% of best substrate
NL-D	SEVNLDAEFR	Swedish mutant APP β-cleavage site	100
EL-D	SEVELDAEFR	Modified APP β-cleavage site	87
DA-A	VVEVDAAVTP	Artifical cleavage site at APP carboxyl terminus	24
NL-A	SEVNLAAEFR	Modified APP β-cleavage site	16
EF-A	SEVEFAAEFR	Modified APP β-cleavage site	15
KM-D	SEVKMDAEFR	Wild-type APP β-cleavage site	9
GY-E	HDSGYEVHHQ	Alternative APP β-cleavage site	8
EF-D	SEVEFDAEFR	Modified APP β-cleavage site	2
P4K	SKVNLDAEFR	Modified APP β-cleavage site	2
VL-M	TSVLMAAP	Cathepsin D substrate (octameric peptide)	2
KL-V	VHHQKLVFFA	APP α-secretase cleavage site	1
HL-V	IHPFHLVIHN	Renin substrate	0

spected under the fluorescence microscope (Leica MZ12 equipped with Leica 2 video system). Brightly fluorescent beads were isolated and submitted to Edman sequencing. The sequencing was performed on a PE Biosystems Procise 494 HT Sequencer.

Portions of 200–400 beads of the six libraries, Lys(dabsyl)-SEXNL-DAEFR-Glu(Gly-PEGA)-Lucifer Yellow, Lys(dabsyl)-SEVXLDAEFR-Glu(Gly-PEGA)-Lucifer Yellow, Lys(dabsyl)-SEVNXDAEFR-Glu(Gly-PEGA)-Lucifer Yellow, Lys(dabsyl)-SEVNLXAEFR-Glu(Gly-PEGA)-Lucifer Yellow, Lys(dabsyl)-SEVNLDXEFR-Glu(Gly-PEGA)-Lucifer Yellow, and Lys(dabsyl)-SEVNLDAXFR-Glu(Gly-PEGA)-Lucifer Yellow, were extensively washed and incubated in a volume of 0.1 ml as described above. Then the beads were washed with the incubation buffer and 100 mM MES buffer, pH 4.8, containing 5% glycerol and 0.05% Triton X-100. Each portion of beads was inspected separately, and the exact number of total beads as well as brightly fluorescent beads was counted. Fluorescent beads were submitted to sequencing.

RESULTS

Cloning, Expression, and Purification of Enzymes—Full-length BACE, BACE ectodomain, and full-length BACE2 constructs were made containing a carboxyl-terminal hexahistidine tag to facilitate purification. The purification process was monitored by Western blotting using an anti-6×His antibody. The presence of enzymatically active β-secretase was checked by a mass spectrometry assay (17), and the enzymatic purity (=absence of contaminating proteases) was determined by a FRET assay using four homologous substrates. The main criteria for purity were the absence of activity with the KL-V and VL-M substrates and presence of activity with the KM-D and NL-D substrates. A further criterion was the preferential cleavage of NL-D over KM-D (see Table I).

Using the purification procedure described under "Experimental Procedures," essentially homogeneous full-length BACE or BACE ectodomain were obtained (Fig. 1). Amino-terminal sequence analyses showed the both full-length BACE (ETDEEPEE) and BACE2 (ALEPALASPA) are efficiently processed to the mature enzymes in the baculovirus/Sf9 expression system. The respective amino-terminal sequences are identical to those observed by other groups (3, 9, 18, 19).

BACE ectodomain (TQHGIRL) is secreted from Sf9 cells as the proenzyme. This is in contrast to the situation in mammalian cell lines where three other groups report efficient processing of the ectodomain using either HEK293 or Chinese hamster ovary cells (20–22). Sf9 cells clearly contain a furin-like protease that is potentially capable of processing the ectodomain since the full-length enzyme is correctly processed in this system. It may be that the soluble ectodomain does not come into extended contact with the furin-like convertase if this protein is, in analogy to furin, membrane-bound. It may also be the case, however, that the very high expression level of BACE ectodomain completely saturates the convertase. The proenzyme as purified here shows almost identical catalytic
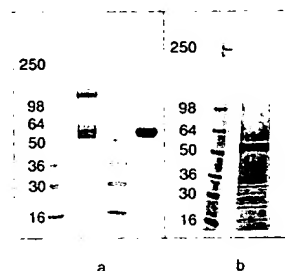

FIG. 1. **Purification of BACE, BACE ectodomain, and BACE2.** The SDS gels stained with Coomassie Blue show (*a*) *left*, full-length BACE and *right*, BACE ectodomain (*b*) BACE2. Both BACE versions show an apparent molecular mass ~52 kDa. Full-length BACE, in contrast to BACE ectodomain, shows an additional band at 110 kDa, which corresponds to dimer (as identified by Western blot, data not shown). BACE2 migrates like BACE.

activity to the mature enzyme, a phenomenon already documented by others, implying that BACE is not a true zymogen (20). The purified BACE ectodomain crystallized and the structure was solved to 2.8-Å resolution.[2] BACE2 was not purified to homogeneity but was shown to be almost free of other contaminating protease activities by analysis with a series of aspartic protease substrates (Fig. 3).

Validation of the Enzyme Assays—The FRET assay was based on dodecameric peptides containing 10 amino acids of the putative substrate and a linker amino acid at each end. The linker amino acids, lysine at the amino terminus and glutamine at the carboxyl terminus, are used to couple the fluorescent group Lucifer Yellow and the quenching group dabsyl to the substrate (Fig. 2). Because the quenching group is coupled via the ε-amino group of lysine, the peptide can still be analyzed by Edman degradation. The substrates were either used as soluble entities or immobilized to beads.

The sequences of the substrates are given in Table I. Most of the substrates cover the β-cleavage site of APP. Some were generated based on the putative specificity of β-secretase (*e.g.* KM-D, NL-D, or GY-E), some to confirm the results obtained by investigating the bead libraries (*e.g.* EL-D) and some as controls (*e.g.* KL-V) or to investigate other proteases (HL-V). Finally, a substrate that is differentially cleaved by BACE and cathepsin D was produced as well (DA-A). This sequence derives from the carboxyl terminus of APP. *In vivo*, β-secretase does not have access to the carboxyl-terminal end of APP since the catalytic domain of the enzyme faces the lumenal side of the membrane, whereas the carboxyl-terminal end of APP is cytosolic. We became aware of a possible *in vitro* cleavage of that
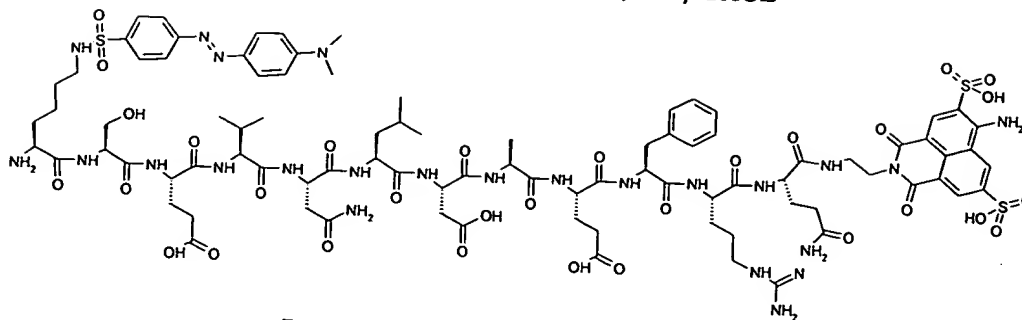
[2] A. Ruf, unpublished data.

Fig. 2. **Structure of the NL-D FRET substrate.**

sequence element by cathepsin D or BACE when we were trying to isolate proteases with β-secretase activity using a mass spectrometry-based enzyme assay (17). In addition to the peptide fragment with the sequence DAEFRHHDSGYEVH-HQK with a mass of 1955 Da, two additional fragments with masses of 1338 and 1267 were identified. These masses correspond to the fragments AAVTPEERHLSK and AVT-PEERHLSK, respectively. Purified BACE generated the fragments with mass 1955 and 1267, whereas cathepsin D generated peptides with masses of 1955 and 1338. As expected, the FRET substrates were cleaved as predicted by the mass spectrometry assay. A selection of identified cleavage sites is given in Table II. They were determined by incubation of soluble substrates by the enzymes followed by separation of the fragments by reversed-phase HPLC and analysis by mass spectrometry.

All FRET substrates used in the present work have a strong excess of quenching capacity. This leads to apparently lower reaction rates (Lucifer Yellow liberated per min) at higher the substrate concentrations. The consequences are apparent substrate inhibition and underestimation of the K_m values. By correcting the quenching effect, the pair BACE and substrate DA-A showed a Michaelis-Menten-like behavior. However, BACE with the substrates KM-D and NL-D still showed a component of non-linearity, in particular at low substrate concentration. Therefore, the kinetic constants were determined graphically (Table IV). Under identical conditions, full-length BACE behaved more Michaelis-Menten-like than BACE ectodomain (data not shown). The origin of this phenomenon is not clear. The two enzyme forms also differ in terms of their stability, with the ectodomain losing activity rapidly on storage at 4 °C. To check if the two enzyme forms are comparable at all, the IC_{50} value for a number of inhibitors was measured in separate experiments with full-length BACE or the ectodomain. The inhibitors were all statin analogues of the OM99-2 and P10-P4' StatVal, covering IC_{50} values between 50 nM and 50 μM. The same order of potency was observed in both cases.[3]

Catalytic Properties of BACE—To compare the preferred substrates and the pH optima for all aspartic proteases at hand, each enzyme was tested with seven substrates in the pH range 2–7.5 at a constant concentration of 10 μM (Table III). A subset of these data is shown in Fig. 3. BACE and BACE2 show a very similar substrate preference differing only with respect to the GY-E and the KL-V-substrates. Both cathepsin D and cathepsin E cleave Swedish mutant APP at the β-site, although with very low efficiency compared with their best substrates. Interestingly, cathepsin D cleaves the APP-derived substrates at a noticeably lower pH optimum than its preferred substrate VL-M (Fig. 3c).

The highest substrate turnover (K_{cat}) of BACE is observed with the NL-D substrate (Table IV). The highest substrate affinity (K_m) value, however, is observed with the DA-A sub-

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[3] E. Kitas, unpublished results.

TABLE II
Identification of the cleavage sites

These experiments were performed in 100 mM sodium acetate buffer using the optimal pH determined for each enzyme (Fig. 3 and Table III). Note that both cathepsin D and E cleave the substrate KL-V between Phe-Phe, which corresponds to position 19–20 in $A\beta$.

Enzyme	Substrate	Cleavage site
BACE	NL-D	SEVNL ↓ DAEFR
	DA-A	VVEVDA ↓ AVTP
	KM-D	SEVKM ↓ DAEFR
Cathepsin D	GY-E	HDSGY ↓ EVHHQ
	VL-M	TSVL ↓ MAAP
	KL-V	VHHQKLVF ↓ FA
Cathepsin E	DA-A	VVEVD ↓ AAVTP
	KL-V	VHHQKLVF ↓ FA
Napsin A	VL-M	TSVL ↓ MAAP
Pepsin	VL-M	TSVL ↓ MAAP

strate. Both the V_{max} value observed with the NL-D substrate as well as the K_m value observed with the DA-A substrate appear less favorable than the corresponding values for cathepsin D measured with its favorite substrate VL-M.

Substrate Specificity of BACE—Several specific peptide libraries were designed to examine the substrate specificity of BACE at and around the β cleavage site in APP. Fluorogenic peptide libraries coupled to a solid phase (polymer beads) were synthesized according to Rossé *et al.* (24). Proteolytic cleavage of bead-linked peptide causes the bead to fluorescence. Fluorescent beads can be manually separated from non-fluorescent beads using a fluorescent microscope. The peptide sequence associated with each fluorescent bead can be directly determined by Edman degradation. BACE ectodomain was used in these experiments after it was observed that the full-length enzyme does not cleave bead-bound substrates. Presumably full-length BACE is too large to be able to penetrate the pores of the beads. The results obtained from the evaluation of several libraries are shown in Table V.

The P2-P1 library was designed to assess which amino acids other than those found in wild-type APP (Lys, Met) and Swedish mutant APP (Asn, Leu) can be accepted in the P2 and P1 positions. The Asn-Leu motif was detected several times, and Leu was also found in combination with Glu or Asp. On the other hand, neither the Lys-Met motif nor anything resembling it was ever detected, indicating that, in our *in vitro* assay at least, wild-type APP is not a preferred substrate for BACE. The Gly-Tyr motif, corresponding to the alternative cleavage site in APP (leading to $A\beta_{11-x}$), was also never detected in these experiments.

Because 22 of 25 experiments revealed Leu as the preferred P1 residue, we generated a second library with a fixed Leu at P1 and variable amino acids at P2 and P1' positions. Asn was found as the preferred residue in P2. From a total of 66 beads, 24 showed Asn, 14 Glu, and 12 Asp, with Gln found once. The hydroxylamino acids Thr and Ser were found at position P2 only in combination with Ser at P1'.

The relatively loose substrate specificity of BACE was fur-

TABLE III
Relative activities of seven aspartic proteases

The numbers were generated by experiments of the type shown in Fig. 3 using the same assay conditions; full-length BACE and all other enzymes as described under "Experimental Procedures," substrate concentrations of 10 μM and a pH gradient from 2 to 7.5 made with 100 mM sodium citrate. The highest activity with the preferred substrate at the pH optimum was set 100%. Experimental details are given in the legend to Fig. 1. BACE-2 was assayed in the presence of 5 μM pepstatin to inhibit possible contaminating aspartic proteases. Cat, cathepsin.

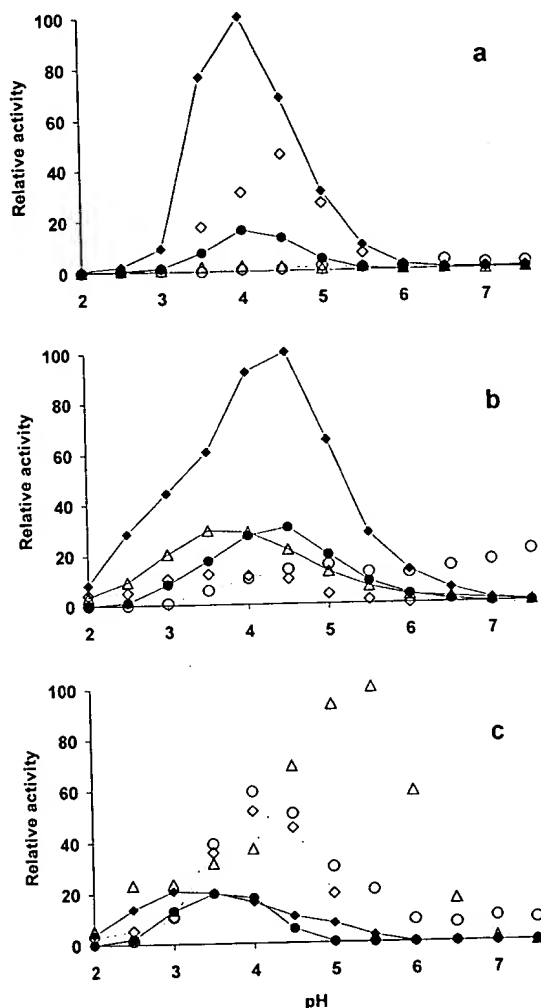| Substrate	BACE pH 4	BACE-2 pH 4	Cat E pH 5	Cat D pH 5	Pepsin pH 4	Napsin A pH 6.5	Renin pH 7
KM-D	9	24	0	0	7	0	0
NL-D	100	100	0	3	4	0	0
KL-V	1	19	100	37	7	8	3
VL-M	2	19	8	100	100	11	100
HL-V	0	0	1	0	-1	0	0
GY-E	8	3	1	1	0	0	0
DA-A	24	32	-1	31	2	0	0

FIG. 3. **Substrate specificity and pH optimum.** The activity of the seven aspartic proteases with the various substrates was determined using 100 mM citrate in the pH range 2–7.5. Shown are the examples of BACE (*a*), BACE2 (*b*), and cathepsin D (*c*). For clarity, only a selection of substrates are shown: ●, KM-D; ◆, NL-D; ○, KL-V; △, VL-M; ◇, DA-A. The highest value was selected as an internal reference and set 100% (see Table III). BACE2 was tested in the absence of pepstatin, in contrast to the experiment shown in Table III. There is an increment of activity toward the substrate VL-M that can be suppressed by pepstatin and, thus, might be due at least partially to a contaminating cathepsin D-like protease. On the other hand, the activity toward the substrate KL-V could not be inhibited by pepstatin, AEBSF, aprotinin, E-64, EDTA, or leupeptin. Noteworthy is the different pH optimum of cathepsin D with respect to the different substrates.

TABLE IV
Kinetic constants of full-length BACE

The values were determined graphically since in the case of the KM-D and NL-D substrates, non-linear inverse plots were observed, thus shifting the V_{max} and K_M values away from any realistic number. Only in the case of the DA-A substrate were the graphically and the mathematically determined values in agreement.

Substrate	Constants	BACE	Cathepsin D
KM-D	K_m (μM)	7	
	V_{max} (nM LuYe/mg/min)	5	
	K_{cat} (s^{-1})	0.002	
	K_{cat}/Km (s^{-1} μM^{-1})	0.0003	
NL-D	K_m (μM)	9	3
	V_{max} (nM LuYe/mg/min)	25	70
	K_{cat} (s^{-1})	0.02	0.04
	K_{cat}/K_m (s^{-1} μM^{-1})	0.002	0.015
DA-A	K_m (μM)	1	2.5
	V_{max} (nM LuYe/mg/min)	2	60
	K_{cat} (s^{-1})	0.004	0.04
	K_{cat}/K_m (s^{-1} μM^{-1})	0.002	0.02
VL-M	K_m (μM)		2
	V_{max} (nM LuYe/mg/min)		1600
	K_{cat} (s^{-1})		1
	K_{cat}/K_m (s^{-1} μM^{-1})		0.5

with Leu as the only amino acid identified in 10 sequencing experiments. Hydrophobic side chains are preferred in the P3 position. Asp and Glu are preferred at P4 and P1'. Positions P2 and P2', on the other hand, tolerate a variety of residues.

To verify these findings, a series of homologous substrates was synthesized (Table I). Replacement of Glu at P4 by Lys of the Swedish mutant substrate (SW) drastically reduced the preference of the enzyme for this substrate, whereas replacement of the Asn at P2 by a Glu had a relatively modest effect. Replacement of the Leu at P1 by Phe reduced the cleavage efficiency to about 10% compared with the SW substrate.

Relation to Other Proteases—The two BACE inhibitors described so far, P10-P4' StatVal and OM99-2, contain the peptide sequence of the APP β-cleavage site whereby the cleavage site is modified to mimic the transition state of the substrate. Both peptides inhibit BACE or BACE2 in the nanomolar range. However, the two peptides inhibit cathepsin D, cathepsin E, and pepsin as well at even lower concentrations (Table VI). P10-P4' StatVal is even more potent toward cathepsin D, cathepsin E, and pepsin than toward BACE, perhaps because its sequence element Sta-Val better mimics the cleavage site between two bulky hydrophobic residues. Inhibitor OM99-2 mimics the sequence Leu-Ala-Ala and is therefore close to substrates NL-A (Table I). Pepstatin, the renin inhibitor Remikiren, and the human immunodeficiency virus-protease inhibitor Saquinavir do not inhibit BACE. The least specific inhibitor appears to be pepstatin and the most specific inhibitors appear to be Remikiren and Saquinavir.

Based on these results and the substrate specificity data, the seven aspartic proteases can be grouped into three clusters, (*a*)

ther shown with a set of six libraries, each containing a random single amino acid at position P4, P3, P2, P1, P1', and P2', respectively (Table V). Again, P1 is the most restricted position

<center>TABLE V</center>
<center>*Substrate preference of BACE ectodomain*</center>

The experiments were performed with a random library of immobilized substrates and the BACE ectodomain. Similar experiments with the full-length enzyme did not reveal any cleavage, probably because the full-length enzyme is too large to enter the pores of the beads. The library type defines the amino acid positions, which were randomly occupied by all natural amino acids except cysteine. "Frequency" gives the number of beads with a particular amino acid sequence. The total number of selected and sequenced beads is calculated by adding these numbers; for instance, the number for the P2-P1 library is 25.

Library type	Sequence	Frequency	Library type	Sequence	Frequency	Library type	Sequence	Frequency
P2-P1	SEV*XX*DAEFR		P4	S*X*VNLDAEFR		P1	SEVN*X*DAEFR	
	NL	9		E	5		L	10
	EL	6		D	4			
	DL	5		G	1	P1'	SEVNL*X*AEFR	
	EF	3					E	4
	QL	1	P3	SE*X*NLDAEFR			D	2
	YL	1		V	5			
				L	4	P2'	SEVNLD*X*EFR	
P2-P1'	SEV*X*L*X*AEFR			I	2		A	4
	NLE	7		T	2		E	3
	ELD	7					V	2
	DLD	7	P2	SEV*X*LDAEFR			T	1
	TLS	6		N	5			
	NLA	5		D	3			
	ALD	4		E	2			
	NLD	3		Y	1			
	QLD	1		M	1			
	SLS	1		A	1			

<center>TABLE VI</center>
<center>*Inhibitor profile of six aspartic proteases*</center>

The IC_{50} values were determined in 100 mM sodium acetate buffer at a substrate concentration of 5 μM and at the optimal pH for the enzyme-substrate pair. Full-length BACE was used.

Enzyme	Substrate (5 μM)	pH	OM99–2	P10-P4'StatVal	Pepstatin	Remikiren	Saquinavir
			μM	μM	μM	μM	μM
BACE	NL-D	4.5	0.08	0.2	80	>100	>100
BACE2	NL-D	4.5	0.12	0.3	10	>100	>100
Cathepsin E	KL-V	4.5	0.01	0.01	0.0007	5	7
Pepsin	VL-M	4.5	0.06	0.006	0.002	38	17
Cathepsin D	VL-M	5.5	4	0.04	0.0007	1	0.2
Renin	HL-V	7	>100	>100	>100	0.013	>100

BACE and BACE2, (*b*) cathepsin D, cathepsin E, napsin A, and pepsin and (*c*) renin. Group *a* prefers leucine at P1 and accepts polar or even acidic residues at P2 and P1'. Position P3 is mostly occupied by a hydrophobic residue, preferentially a valine residue. Group *b* prefers bulky hydrophobic residues at positions P2, P1, and P1', and group *c*, represented by renin, appears to be very specific, with the angiotensinogen cleavage motif the only known substrate. Groups *a* and *c* appear to be the most distant enzymes with respect to pH optimum, substrate specificity, and cross-reactivity of the inhibitors. In this respect it is perhaps unsurprising that not a single BACE inhibitor was found in a library containing more than1800 renin inhibitors.[4]

<center>DISCUSSION</center>

The results of the present investigation can be summarized as follows: (*a*) BACE exhibits very poor kinetic constants when assayed with soluble decameric peptides deriving from the APP β-cleavage site (*b*) BACE accepts a wide variety of peptidic substrates and, in contrast to other mammalian aspartic proteases, prefers acidic or polar residues at the P2 and P1' positions (*c*) an alternative substrate, DA-A, which is unrelated to the β-secretase cleavage site in APP, was identified.

BACE exhibits very low catalytic efficiency toward its most preferred substrate (K_{cat}/K_m = 0.002 for NL-D). The catalytic efficiency of cathepsin D, for instance, with the best substrate tested here, is 250-fold higher (K_{cat}/K_m = 0.5 for VL-M). Similar values for BACE have been reported by other groups using a

variety of BACE constructs expressed in diverse cellular expression systems and with other FRET substrates (5, 23, 25, 26). The poor performance of BACE is therefore unlikely to be because of the quality of the protein. It may be that APP is not the only substrate for BACE *in vivo* and that a better, as yet unidentified substrate, would give a much higher K_{cat}/K_m value. However, the peptidic substrate analysis described here indicates that the Swedish mutant APP peptide, NL-D, already contains an optimal sequence of amino acids in the P4-P2' positions so it seems unlikely that a better substrate exists. There may be factors other than the peptide sequence itself that contribute to the catalytic efficiency of BACE. Our data show that the KM-D peptide, which derives from the BACE cleavage site in wild-type APP, is an extremely poor substrate, but a number of publications document that BACE cleaves wild-type APP *in vitro* and *in vivo* (1, 3, 6, 7). The fact that BACE does cleave wild-type APP *in vivo* may have to do with the juxtaposition of these two proteins in the lumenal membrane of the ER-Golgi system. Both the catalytic domain of BACE and the β-secretase cleavage site in APP face into the lumen, and this may provide the optimal orientation for substrate-protease interaction, allowing for more efficient substrate binding and cleavage. A recent publication showing that the transmembrane domain of BACE is essential for efficient interaction of APP and BACE supports this interpretation (27).

The data presented above also indicate that BACE can accept a wide variety of peptidic substrates. Compared with other mammalian aspartic proteases, BACE is more like cathepsin D (relatively unspecific) than renin (highly specific) in terms of substrate specificity. However, BACE clearly has a different

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[4] H. Döbeli, unpublished data.

substrate selectivity from cathepsin D, preferring polar or acid amino acids in the P2 and P1′ positions; cathepsin D and indeed all other mammalian aspartic proteases prefer hydrophobic amino acids in these positions. A more problematic issue for drug developers might be the extended substrate pocket in BACE. Any attempt to decrease the size of the inhibitor OM99-2 resulted in a gradual loss of inhibitory potency regardless of whether amino-terminal or carboxyl-terminal amino acids were deleted.[3]

The limited substrate and inhibitor analysis undertaken here shows that BACE and BACE2 are very similar, at least with respect to their ability to digest the BACE substrates NL-D, KM-D, GY-E, and DA-A. A small distinction between the two enzymes was observed with the cathepsin D/E substrates VL-M and KL-V (Table III and Fig. 3), these substrates being cleaved at a low level by BACE2 but not by BACE. Despite these subtle differences between BACE and BACE2 *in vitro*, a recent publication suggests that BACE2 might not actually cleave at the β-secretase site at all *in vivo*, with the enzyme preferring to cleave further downstream at a second site proximal to the α-secretase cleavage site in APP (28). The reason for this is unclear but it suggests that it might be possible to develop BACE inhibitors that might act selectively *in vivo*.

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